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LIPASE SYSTEMS USED IN THE MANUFACTURE OF ITALIAN CHEESE. II. SELECTIVE HYDROLYSIS[1,2]

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Crude lipase preparations selectively hydrolyzed, at different rates, individual fatty acids from triglycerides. Pancreatic lipase liberated C-12 fatty acids or higher, that from Aspergillus niger released the lower ones, and milk lipase released both lower and higher acids. Lipases used in manufacturing Italian cheese hydrolyzed much butyric acid which varied with the enzyme source. Those from the same gland but from different animals varied in their lipolytic activity. *Editor.*

The proportions of individual free fatty acids in ripened cheese are known to influence its flavor characteristics. In certain cheeses, such as Romano and Provolone, where an active lipase is added to the milk prior to coagulation, any difference in selectivity in the hydrolysis of specific fatty acids from milk fat would influence flavor. Indirect evidence of selective hydrolysis has been indicated in studies which showed that the rate of butyric acid liberation in both Provolone and Romano cheese was related to the animal source of the lipase preparation *(4, 6)*. More direct evidence of selective hydrolysis by lipases has been reported recently *(1, 7, 9, 11)*.

Shipe *(11)* found that different ratios of butyric and caprylic acids were liberated from equimolecular mixtures of tributyrin and tricaprylin by lipases of *Aspergillus niger* and *Penicillium roqueforti*. Wilcox *et al. (12)* demonstrated that different ratios of lower fatty acids were liberated when various microorganisms hydrolyzed milk fat. Exploratory research on lipases used in Italian cheese manufacture revealed differences in the relative amounts of butyric acid in the fatty acid fraction after lipolysis of milk fat by these lipases *(1)*.

This paper presents a continuation of studies on the selective hydrolysis of fatty acids. Such information should be valuable both in adding to the knowledge of the cheese-ripening process and as a means of evaluating lipase preparations for cheese manufacture and other food uses.

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EXPERIMENTAL PROCEDURE

The selective hydrolysis of fatty acids from fat by the various lipase systems was determined by measuring the rates of hydrolysis of synthetic triglycerides, and by determining the differences in the end-products of milk fat hydrolysis. The previous study in this series showed differences in 11 lipase systems used in Italian cheese manufacture *(3)*.

Based on the results of that study, five of the same enzyme preparations were studied. They were: (*a*) domestic purified calf rennet paste, (*b*) imported crude kid rennet paste, (*c*) calf oral glandular preparation, (*d*) kid oral glandular preparation, and (*e*) lamb oral glandular preparation. In addition, milk lipase, *Aspergillus* lipase, and hog pancreatic lipase were studied.

Methods of determining triglyceride hydrolysis. The reaction mixture was analyzed by chromatographic techniques. The extent of hydrolysis was determined by direct extraction of the total free fatty acids on a buffered silica gel column *(5)*, the extraction of a 5- to 10-ml. sample being made with 250 ml. of 5% butanol in chloroform.

The mole per cent butyric acid was measured by buffered silica gel chromatography *(2)*.

Buffers. The buffers were phosphate at pH 6.6 (0.2 *M*) and phthalate at pH 5.0 (0.2 *M*) (pH levels of milk and cheese, respectively). The strength of the buffers was adjusted to a final concentration of 0.1 *M*.

Substrates. All of the synthetic triglycerides were obtained from Eastman Chemical Company, and were used as the substrates without further purification. The insoluble substrates were emulsified with the buffer in a Waring-type blender. Time of mixing was varied, so that the globule size of the different glycerides was approximately ten microns. The various triglycerides were present in the final reaction mixture in concentrations of 0.1 *M*.

Incubation temperatures were maintained at 32° C.±0.5° C. The incubation time was selected so that the rate of hydrolysis was linear with respect to time. Incubation times reported in this study varied from 0.5 of an hour to 3 hours. The enzymatic activity was proportional to the enzyme concentration, and substrate was always present in excess.

Phospholipase activity of preparations. In initial trials with tributyrin, lecithin (soybean) was used as an emulsifying agent. The tributyrin was emulsified with pH 6.6 buffer and 0.1% lecithin. Results revealed that some of the enzyme preparations contained phospholipases which hydrolyzed fatty acids from the phospholipid molecule. Chromatographic analysis *(2)* revealed that about ten to 20 mole per cent of free acids were "nonbutyric" fatty acids, when the crude kid rennet paste or milk supplied the lipase source. Butyric acid only was found when the glandular enzyme preparations were used. Since some of the preparations contained phospholipase activity, lecithin was not used in further experiments. Samples were shaken during incubation with a Burrell "wrist-action" shaker.

Hydrolysis of synthetic triglycerides. The activity of the various enzyme preparations was determined on tributyrin, tricaproin, tricaprylin, and tripal-

TABLE 1

Hydrolysis of triglycerides by different commercial lipase preparations[a]

Enzyme preparations	Relative hydrolysis					
	Tributyrin	Tricaproin	Tricaprylin	Tricaprin	Trilaurin	Tripalmitin
	(%)					
Domestic calf rennet paste	33	58	17	100	58	65
Imported kid rennet paste	100	68	18	91	12	24
Glandular oral calf preparation	100	66	5	88	24	19
Glandular oral kid preparation	100	31	87	80	32	21
Glandular oral lamb preparation	53	100	55	60	15	8

[a] Average of three trials.

mitin. All studies on the synthetic glycerides were conducted at pH 5.3, which is the average pH level of Italian cheese. The relative hydrolysis of the different triglycerides by the various enzyme preparations is shown (Table 1).

Results are presented as per cent of the glyceride which was hydrolyzed at the fastest rate by each preparation (the glyceride hydrolyzed at the fastest rate was arbitrarily set at 100%). Generally, tributyrin was more rapidly hydrolyzed than the other triglycerides. However, there was a definite difference in the relative rates of hydrolysis by the different preparations. For example, although the glandular calf and kid preparations hydrolyzed tributyrin more rapidly than other glycerides, the kid preparation hydrolyzed tricaprylin 17 times as fast as did the calf preparation. The imported rennet paste and glandular calf preparation, however, were quite similar in the rates at which they hydrolyzed the various triglycerides.

Method of determining selectivity of milk fat hydrolysis. The incubation temperature, enzyme sources, and buffers were the same as those used for the triglyceride study. The milk fat substrate was in the form of 20% cream which had been homogenized at 2,500 p.s.i. and pasteurized at 170° F. for 30 minutes. Analysis for butyric and total free fatty acids was made by the chromatographic method previously cited *(2)*. Caproic, caprylic, and capric acids were determined by the method of Ramsey and Patterson *(9)*.

Selective liberation of butyric acid from milk fat. The selective liberation of butyric acid from milk fat by various lipase preparations was reported previously *(1)*. These trials, which were conducted under more controlled conditions, confirmed the earlier work for butyric acid. Results revealed that the ratio of butyric to total acid was not always a constant throughout the incubation period. Therefore, it was necessary to establish experimentally when this ratio was constant for each different enzyme preparation. The effect of time on the ratio of butyric and higher acids liberated is illustrated by the representative data in Figure 1, which show the ratio of butyric acid per total acids liberated for five of the enzyme preparations. The results, presented as mole per

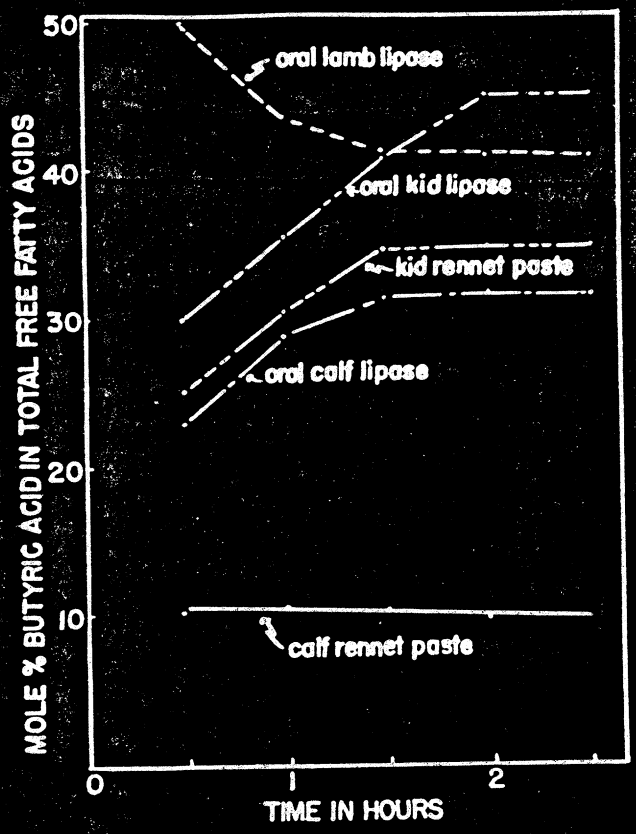

FIG. 1. Effect of incubation time on the relative concentration of butyric acid hydrolyzed from milk fat.

cent butyric acid in the total free fatty acids, showed that in the case of the calf and kid glandular preparations and the kid rennet paste, the butyric acid is released at a faster rate than the higher acids after the first half-hour, but that the relative rate is constant after two hours. The lamb lipase product produced more butyric acid initially during the first 90 minutes, then the ratio remained relatively constant for the next two hours.

The mole per cent butyric acid remained a constant between two and five hours at 37° C. and between five and 20 hours at 5° C. The mole per cent of other fatty acids was constant within the same time limits.

Selective hydrolysis of butyric, caproic, caprylic, and capric acids from milk fat. Further experiments were conducted in which the analyses of free fatty acids were extended to include caproic, caprylic, and capric acids. Conditions in these experiments were identical to those previously described, except for the additional analyses. In addition to the five lipase systems previously studied, freeze-dried raw milk, *Aspergillus* and pancreatic lipase were also investigated. Results are presented (Table 2).

Results for butyric acid are within the range reported previously *(1)*. The relative amounts of the other free acids also varied according to the enzyme

TABLE 2

Selective liberation of individual fatty acids from milk fat by eight different lipase preparations

Lipase source	Butyric	Caproic	Caprylic	Capric	Lauric and higher
	(%)[a]				
Imported crude kid rennet paste	32.8	11.3	7.1	11.8	33.6
Domestic purified calf rennet paste	10.7	3.1	trace	trace	86.5
Calf oral lipase	36.7	8.9	4.8	10.7	39.0
Kid oral lipase	44.4	15.2	7.6	12.3	21.5
Lamb oral lipase	48.1	8.6	14.2	9.3	19.8
Aspergillus lipase	43.1	18.9	20.2	17.5	trace
Milk lipase	13.5	8.2	10.2	8.7	60.0
Pancreatic lipase	8.4	2.1	trace	trace	89.1

[a] Values expressed as per cent of total free fatty acids (on microequivalent basis). Average of five trials. Reaction time not in excess of three hours at 35° C.

source. Pancreatic lipase action resulted in 90 mole per cent of higher fatty acids in the total free fatty acid mixture; whereas, only a trace of these acids was hydrolyzed from milk fat by the *Aspergillus* lipase preparations. Similarly, there is considerable selectivity in the hydrolysis of caproic, caprylic, and capric acids from milk fat. The mole per cent of caproic acid in the total free fatty acids varied from 2.1 to 28.9. The imported kid rennet paste, kid oral lipase, and *Aspergillus* lipase all released more than ten mole per cent caproic acid. Similar variation in amount of caprylic and capric acid present in the free fatty acid mixture was noted.

Some of the preparations are known to contain two or more different lipase enzymes. Schwartz *(10)* demonstrated that the milk lipase system is heterogeneous, and Harper and Gould (*3*) demonstrated two or more lipases present in the three different oral lipase preparations.

Factors affecting selective hydrolysis. Limited studies were made concerning the effect of pH, the temperature of the reaction on the selective hydrolysis of butyric acid from milk fat, and the effect of partial heat inactivation. The calf and kid glandular preparations were chosen for these studies, because they were shown to possess different lipase systems. The calf lipase had pH optima at about 5.3, 6.1, and 7.6; whereas, the kid lipase had pH optima at about 5.9 to 6.2 and about 8.6 *(3)*.

Studies were made at pH 5.3 and 6.6 since these values represent the pH of cheese and milk, respectively. The samples were adjusted to their respective pH levels prior to heating. Temperature used for inactivation was selected as based on temperatures used in treatment of Provolone curd. Results in Table 3 show the effect of partial heat inactivation (140° F. for 15 and 30 minutes), reaction temperatures, and pH on the selective liberation of butyric acid.

Results show that the kid lipase hydrolyzed the same relative amount of butyric acid from the fat regardless of the pH, reaction temperature, or degree of heat inactivation. However, the calf lipase showed different butyric acid values at pH 5.3 and pH 6.6. The mole per cent butyric acid (at pH 5.3) was

TABLE 3

Effect of various environmental factors on the selective hydrolysis of butyric acid from milk fat [a]

Time heated at 60° C.	pH 6.6			pH 5.3		
	Heat inactivation of lipase	Butyric acid		Heat inactivation of lipase	Butyric acid	
		5° C.	35° C.		5° C.	35° C.
(min.)	*(%)*	*(mole %)*		*(%)*	*(mole %)*	
		Kid lipase				
0	0	39.7	39.5	0	38.6	39.4
15	45	38.7	39.3	12	39.7	39.2
30	76	39.2	38.0	20	39.1	39.0
		Calf lipase				
0	0	32.6	34.5	0	37.8	39.8
15	56	31.2	33.6	27	34.7	36.7
30	82	30.7	32.5	48	36.5	35.3

[a] Figures are averages of two trials. Reaction time was two hours at 35° C. and five hours at 5° C.

about the same as for the kid lipase. The data also revealed slight but consistent changes in the relative amount of butyric acid when the reaction temperature was changed or when some heat inactivation had occurred. Differences in the two enzyme preparations might be related to differences in the lipases present in the two preparations, to differences in inhibitors present, or to the action of other enzymes. The last possibility cannot be ignored, since lower fatty acids, especially butyric acid, can serve as the substrate for other enzyme systems. If enzyme systems were present in the crude preparations which could utilize the fatty acids, then the results could be an index of the selective utilization of the various fatty acids as well as indicating selective hydrolysis.

DISCUSSION

The selective release of fatty acids from milk fat by different lipase preparations has been demonstrated in this study. However, interpretation of these findings in regard to the reason or mechanism of the selection is not possible at this time. All of the lipase preparations were crude materials containing numerous other materials in addition to lipases. The possible presence of other enzymes, inhibitors, or activators could conceivably influence the relative proportions of the various acids in the total free fatty acid mixture.

In addition, the selective utilization of specific acids might influence the results. However, analyses revealed the complete absence of any detectable concentration (less than 0.1 mg./100 ml. milk) of Beta-keto acids, indicating lack of Beta-oxidation of fatty acids. However, this is insufficient evidence to justify final conclusions.

Since the completion of this work, Mattson *(8)* has reported evidence to show that crude pancreatic lipase is substrate specific in its action, in regard to the position of the fatty acid on the triglyceride molecule. In view of this report, it is conceivable that the selectivity of hydrolysis from a complex fat such as

butter fat is an actual indication of true substrate specificity. Proof of this hypothesis will depend on further investigation with relatively pure lipase preparations.

The findings of this study have considerable practical significance. They provide a basis for a sound explanation of the failure of some lipase systems to produce satisfactory flavor in Italian cheese. In addition, the findings suggest a tool for use in evaluating lipase systems for flavor production. By knowing the end-products desired, analyses can be made on the acids released from the substrate to be used. By selecting a preparation that produces free fatty acids in similar proportion to that desired, a manufacturer can eliminate guesswork in his lipase selection. Similarly, lipase selections can be made for other food products, such as in the manufacture of milk powder for milk chocolate manufacture.

The fact that heat treatment had little if any effect on the selectivity of fatty acid hydrolysis is significant to the manufacture of Italian cheese, especially Provolone. This means that the cheese manufacturer will obtain the same type of lipase action in Provolone as in Romano. Therefore, he will need to correct only for loss of total lipase activity.

SUMMARY

Various crude lipase preparations were shown to hydrolyze triglycerides at different rates. Analysis for free fatty acids released by the hydrolysis of milk fat revealed that these same lipase preparations were selective in the individual free fatty acids hydrolyzed from the same substrate, under the same experimental conditions. The type of acids predominating in the free fatty acid mixture varied with the enzyme preparation. Pancreatic lipase hydrolyzed predominantly fatty acids of C-12 or higher, whereas the *Aspergillus* lipase released primarily the lower ones. Milk lipase released significant concentrations of both higher and lower fatty acids. Lipases used for the manufacture of Italian cheese hydrolyzed relatively high concentrations of butyric acid, which varied with the animal source of the lipase. Lipases from the same gland, but from different animals, produced different relative amounts of free fatty acids.

The use of heat treatment similar to that used for Provolone cheese revealed no effect on selective hydrolysis, and only about 25% loss of lipase activity at the pH of the cheese curd.

The selective hydrolysis of fatty acids from milk fat provides a basis for the selection of lipase preparations for Italian cheese manufacture.

REFERENCES

(1) HARPER, W. J. Apparent Selective Hydrolysis of Butyric Acid from Milk Fat by Various Lipase Preparations. *J. Dairy Sci.*, 38: 1391. 1956.

(2) HARPER, W. J., AND ARMSTRONG, T. V. Measurement of Butyric Acid in Fat with Reference to the Detection of Substitute Fats in Dairy Products. *J. Dairy Sci.*, 37: 481. 1954.

(3) HARPER, W. J., AND GOULD, I. A. Lipase Systems Used in the Manufacture of Italian Cheese. I. General Characteristics. *J. Dairy Sci.*, 38: 87. 1955.
(4) HARPER, W. J., AND LONG, J. E. Italian Cheese Ripening. IV. Various Free Amino and Fatty Acids in Commercial Provolone Cheese. *J. Dairy Sci.*, 39: 129. 1955.
(5) HARPER, W. J., SCHWARTZ, D. P., AND EL-HAGARAWY, I. S. A Rapid Silica Gel Method for Measuring Total Free Fatty Acids in Milk. *J. Dairy Sci.*, 39: 46. 1956.
(6) LONG, J. E., AND HARPER, W. J. Italian Cheese Ripening. V. Various Free Amino and Fatty Acids in Commercial Romano Cheese. *J. Dairy Sci.*, 39: 138. 1956.
(7) MARTIN, H. F., AND PIERS, F. G. Oat Lipase. *Biochem. J.*, 55: 523. 1953.
(8) MATTSON, F. H. Pancreatic Lipase, New Tool for Determining Structures of Triglycerides. *Federation Proc.*, 15: 1834. 1956.
(9) RAMSEY, L. L., AND PATTERSON, W. I. Separation and Identification of Volatile Saturated Fatty Acids (C_5 to C_{10}). *J. Assoc. Off. Agr. Chemists*, 28: 644. 1945.
(10) SCHWARTZ, D. P., GOULD, I. A., AND HARPER, W. J. Raw Milk Lipase System. *J. Dairy Sci.* (In press.)
(11) SHIPE, W. F., JR. A Study of the Relative Specificity of Lipase Produced by *Penicillium roqueforti* and *Aspergillus niger*. *Arch. Biochem.*, 30: 165. 1951.
(12) WILCOX, J. C., NELSON, W. O., AND WOOD, W. A. The Selective Release of Volatile Acids from Butterfat by Microbial Lipases. *J. Dairy Sci.*, 38: 775. 1955.